

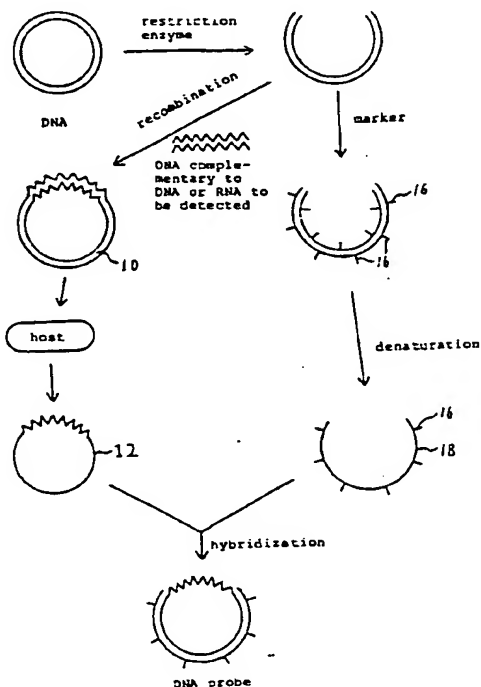


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>4</sup> :</b> <b>C07H 21/04, C12Q 1/68, 1/70</b> <b>// C12N 15/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 87/04165</b> <b>(43) International Publication Date:</b> <b>16 July 1987 (16.07.87)</b>
<b>(21) International Application Number:</b> <b>PCT/JP86/00662</b> <b>(22) International Filing Date:</b> <b>26 December 1986 (26.12.86)</b> <b>(31) Priority Application Number:</b> <b>60/292809</b> <b>(32) Priority Date:</b> <b>27 December 1985 (27.12.85)</b> <b>(33) Priority Country:</b> <b>JP</b> <b>(71) Applicant:</b> <b>TORAY INDUSTRIES, INC. [JP/JP]; 2, Nihonbashi-Muromachi 2-chome, Chuo-ku, Tokyo 103 (JP).</b> <b>(72) Inventors:</b> <b>MURAO, Yasuo ; 1-31-17, Tsunishi, Kamakura-shi, Kanagawa 248 (JP). HOSAKA, Shuntaro ; 3865, Jindaiji, Mitaka-shi, Tokyo 181 (JP). MIURA, Kumiko ; 3-5-18, Daigiri, Fujisawa-shi, Kanagawa 251 (JP).</b>	<b>(74) Agent:</b> <b>TANIGAWA, Hidejiro; 6F, Iwata Bldg., 4-5-12, Iidabashi, Chiyoda-ku, Tokyo 102 (JP).</b> <b>(81) Designated States:</b> <b>AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</b> <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

**(54) Title:** DNA PROBE AND METHOD OF PREPARING THE SAME**(57) Abstract**

A DNA probe with high sensitivity which is easy to handle. The DNA probe of the present invention comprises a single stranded DNA fragment which is complementary to a DNA or RNA to be detected, and a double stranded DNA fragment having a non-radioactive marker or a functional group to which a non-radioactive marker can be attached.



***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

- 1 -

## D E S C R I P T I O N

## DNA PROBE AND METHOD OF PREPARING THE SAME

TECHNICAL FIELD

5           This invention relates to a DNA probe which is used for detecting or quantifying a DNA or RNA originated from a virus, microorganism, plant or animal cell, or the like.

BACKGROUND ART

10           The base sequence of a DNA or RNA is unique to the virus or the organism containing the DNA or RNA. The DNA or RNA hybridizes with a DNA or RNA which is complementary thereto to form a double strand. Recently, utilizing this property, DNA probes are used for  
15   detecting or quantifying DNAs and RNAs.

          Heretofore, DNA probes are prepared by directly labelling a DNA or RNA complementary to a DNA or RNA of a virus, microorganism or plant or animal cell to be detected with a label. The most sensitive labels are  
20   radio labels. However, the radio labels have the drawbacks in that the more sensitive, the shorter the half life period, that it is dangerous to handle, and that a special and expensive equipment is required. Thus, it is desired to label the probe with a non-radioactive  
25   marker.

          Recently, enzyme labels utilizing biotin-avidin bond are used. Avidin is a basic protein with a molecular weight of 68,000 which is contained in egg white, and it has a high affinity with biotin with a molecular weight  
30   of 244, the affinity constant being as high as  $10^{15} \text{M}^{-1}$ . Labelling with an enzyme is conducted by labelling a DNA probe complementary to a DNA or RNA to be detected with biotin which does not hinder very much the hybridization of the DNA probe with the DNA or RNA to be detected  
35   because of its low molecular weight, and then binding an avidin-enzyme conjugation with the biotin on the DNA

- 2 -

probe by means of avidin-biotin bond after the DNA probe is hybridized with the DNA or RNA to be detected.

The known methods for labelling a DNA with biotin includes nick translation method in which a nucleotide  
5 constituting the DNA is replaced with a biotin-conjugated nucleotide in the presence of deoxyribonuclease and DNA polymerase; and a method in which photobiotin (commercially available from BRESA Inc.) is reacted with DNA under irradiation of light.

10 Antigen-antibody reaction is also utilized for labelling DNA probes. In this method, the DNA probe is first labelled with a hapten such as biotin, fluorescein and N-acetoxy-2-acetylaminofluorene, and after the hybridization with a DNA or RNA to be detected, an  
15 antibody specific to the hapten bonded to the DNA probe, which antibody is labelled with an enzyme or a fluorescent substance, is complexed with the hapten on the DNA probe to detect the DNA or RNA to be detected.

Most of the conventional DNA probes except for those  
20 chemically synthesized are double stranded. Thus, when the DNA probe is hybridized with a DNA or RNA to be detected, the DNA probe must be denatured to single strand by an alkali or heat treatment. Further, since the DNA itself which is complementary to the DNA or RNA to be  
25 detected is labelled, the complementarity is degraded, so that the hybridization is interfered to decrease the detection sensitivity. Especially, if the DNA probe is directly labelled with a large molecular substance such as an enzyme, the hybridization is severely hindered.

30 Further, a DNA or RNA originated from a source different from that of the DNA or RNA to be detected is often contaminated in the test sample. In cases where a DNA produced by using a vector is used as the DNA probe, the DNA region derived from the vector is not usually  
35 eliminated sufficiently. Therefore, if the test sample contains as a contaminant a DNA or RNA originated from

- 3 -

the same source as the vector, the DNA or RNA is detected to bring about a false positive result.

#### DISCLOSURE OF THE INVENTION

Accordingly, the object of the present invention is to provide a DNA probe with a high detection sensitivity, which is safe to handle and which is simply used.

This and other objects of the present invention are accomplished by providing a DNA probe comprising a single stranded DNA fragment which is complementary to a DNA or RNA to be detected, and a double stranded DNA fragment having a non-radioactive marker or a functional group to which a non-radioactive marker can be attached.

According to the present invention, since the double stranded region which does not participate in the hybridization with the DNA or RNA to be detected is labelled and so the DNA fragment complementary to the DNA or RNA to be detected is in its inherent state, the hybridization is not interfered by the label at all, so that the detection sensitivity is high. Further, since the region other than the DNA fragment which is subjected to the hybridization with the DNA or RNA to be detected is double stranded and does not hybridize with any DNA or RNA, even if a DNA or RNA of the same origin as the double stranded region of the DNA probe is contained in the test sample, the contaminant does not hybridize with the DNA probe, so that false positive result is not obtained. Since the region of the DNA probe of the present invention, which region is complementary to the DNA or RNA to be detected, is single stranded, there is no need to denature the probe before use, so that it is simply used. Since the DNA probe of the present invention does not utilize a radioactive label, the handling of the probe is safe and no special equipment is necessary. In cases where the double stranded region of the DNA probe of the present invention contains the functional group to which a non-radioactive marker can be attached, the DNA

- 4 -

probe can be directly labelled with an enzyme. This not only offers convenience, but also makes it possible to identify an unknown DNA or RNA by using a mixture of the DNA probes of the present invention, each of which is labelled with a different marker.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic view for explaining a method of preparing the DNA probe of the present invention; and

Fig. 2 is a schematic view for explaining another method of preparing the DNA probe of the present invention.

#### BEST MODE FOR CARRYING OUT THE INVENTION

As stated above, the DNA probe of the present invention has a single stranded portion which is complementary to DNA or RNA to be detected. The source of the DNA or RNA to be detected by the DNA probe of the present invention includes, for example, viruses such as hepatitis virus (A and B), AIDS virus (HIV-III), ATL virus (HIV-I), herpes simplex virus (type 1 and 2), cytomegalovirus, rubeola virus, rubella virus, poliovirus, coxsackie virus, echovirus, influenza virus, rabies virus, yellow fever virus, Japanese encephalitis virus, Marburg disease virus, adenovirus, dengue virus, EB virus, mumps virus, vaccinia virus, Parvovirus, Papovavirus, Rotavirus, Tanapoxvirus, Yabavirus, Lassa virus, tobacco mosaic virus; mycoplasmas; rickettsiae such as Rickettsia tsutsugamushi, Q fever rickettsia, Rickettsia prowazekii, Chlamydia trachomatis, Chlamydia psittaci; bacteria such as Neisseria gonorrhoeae, Clostridium tetani, Staphylococcus aureus, Streptococci, tubercle bacillus, Bacillus anthracis, Diplococcus pneumoniae, Salmonellae, Vibrio cholerae, Salmonella typhi, Salmonella paratyphi, Clostridium botulinum, Brucellaceae, dysentery bacillus, plague bacillus, Escherichia coli, Campylobacters; yeast such as Candida; Protozoa such as Toxoplasma, Plasmodium, Spirochaetales

- 5 -

such as Treponema; animal and plant cells such as tumor cells and cancer cells. The DNA or RNA to be detected may have the whole base sequence or have only a part of the sequence, and may be single stranded or double stranded.

5       The DNA fragment complementary to the DNA or RNA to be detected is usually one originated from the same source as the DNA or RNA to be detected. It should be noted however, any DNA or RNA including those extracted from the source virus, bacterium, microorganism or plant  
10 or animal cell; those produced by genetic engineering technique in which the DNA or RNA originated from the source is inserted in a vector and the vector is replicated in a host; and those chemically synthesized in cases where the base sequence of the DNA or RNA is known  
15 can be used.

      The non-radioactive markers which may be used in the DNA probe of the present invention includes markers such as fluorescent substances, chemiluminescent substances and enzymes, and further includes substances which can  
20 combine such markers, such as low molecular weight substances including biotin and N-acetoxy-2-acetylaminofluorene, antibodies to which such low molecular weight substances act as haptens, high molecular weight substances such as avidin which can bind  
25 the low molecular weight substances, and conjugates of a marker and the above-mentioned substances. Non-limiting examples of the fluorescent substances include fluorescein and rhodamine. Non-limiting examples of chemiluminescent substances include luminol, isoluminol,  
30 N-(4-aminobutyl)-N-ethyl isoluminol, N-(6-aminoethyl)-N-ethyl isoluminol, N-(4-aminobutyl)-N-ethylisoluminolhemisuccinamide, lophine, lucigenine, acridinium esters, pyrogallol, luciferin, indole, riboflavin,  
35 2-methyl-6-phenyl-3,7-dihydroimidazo(1,2-a)-pyradine-3-one, and derivatives thereof. Non-limiting examples of

- 6 -

enzymes include peroxidase, beta-galactosidase, alkaline phosphatase and acid phosphatase.

Although the DNA may be directly labelled with a high molecular weight marker, it is also possible to  
5 label the DNA with a low molecular weight marker such as biotin and then to attach a high molecular weight substance such as avidin which specifically binds the low molecular weight substance, to which high molecular weight substance a marker such as an enzyme or a  
10 fluorescent substance is conjugated. It is also possible to label the DNA with a hapten and then to attach an antibody-enzyme conjugate or an fluorescence-labelled antibody, which antibody is specific to the hapten.

The functional groups to which a non-radioactive  
15 marker can be attached and known and non-limiting examples thereof include amino group, carboxyl group, mercapto group, hydroxyl group, epoxy group and formyl group. If the DNA has such a group, it can be directly labelled with an enzyme. How to incorporate such groups  
20 into DNA is described, for example, in European patent 63,879 or Nucleic Acid Research 9(8), p.1933 (1981). It should be noted that in cases where the DNA probe of the present invention contains such a functional group, a non-radioactive marker should be attached to the  
25 functional group. The attachment of the non-radioactive marker to the functional group may be conducted before or after the hybridization with the DNA or RNA to be detected.

The double stranded region of the DNA probe of the  
30 present invention may be any DNA which is labelled with a non-radioactive marker or which has a functional group capable of attaching a non-radioactive marker, and to which a single stranded DNA complementary to the DNA or RNA to be detected can be ligated, and may be, for  
35 example, a vector DNA or a synthetic DNA. Among these, those originated from a bacteriophage having a single



- 7 -

stranded circular DNA, such as  $\phi$ X-174, S13, M12, f1, fd and M13 phages are preferred.

The size of the DNA probe of the present invention is not important and may widely vary from 12 bases to several tens kb.

5 The DNA probe of the present invention can be prepared by two fundamental methods. In the first method, a first single stranded DNA containing the fragment complementary to the DNA or RNA to be detected is hybridized with a second single stranded DNA which contains a region complementary to a portion of the first single stranded DNA, which portion is other than the DNA fragment complementary to the DNA or RNA to be detected, the second single stranded DNA having a non-radioactive marker or a functional group to which a non-radioactive marker can be attached. In the second method, a first single stranded DNA containing a single stranded DNA fragment complementary to the DNA or RNA to be detected is provided, and then a complementary DNA strand (hereinafter referred to as second DNA) is formed on a region of the first single stranded DNA, which region is other than the single stranded DNA fragment complementary to the DNA or RNA to be detected, using the region of the first single stranded DNA as a template, and using a nucleotide having a non-radioactive marker or a functional group to which a non-radioactive marker can be attached. In these two methods, in cases where the functional group to which a non-radioactive marker can be attached is used, the non-radioactive marker may be attached after the double stranded DNA is formed.

The above-mentioned two methods will now be described in detail based on preferred embodiments thereof referring to the accompanying drawings.

35 A preferred embodiment of the first method in which a bacteriophage (hereinafter referred to as phage) is utilized will now be described referring to Fig. 1.

- 8 -

Phages, i.e., viruses of which host is a bacterium or a ray fungus are known for long years. Among the phages,  $\phi$ X-174, S13, M12, f1, fd and M13 are known to have a single stranded circular DNA. Upon incorporation  
5 of the DNA of such a phage into the host cell, double stranded circular DNAs called as replication form are first formed, and then single stranded circular DNAs are formed using the double stranded circular DNA as a template, and the single stranded circular DNAs thus  
10 formed are then released from the cell in the form of a phage. The preferred embodiment of the first method utilizes such a phage. First, double stranded circular DNA of the phage is taken from a host cells infected with the phage, and the double stranded circular DNA is then  
15 cut with a restriction enzyme to open the ring. A double stranded DNA complementary to a DNA or RNA to be detected, which is cut with the same restriction enzyme is then recombined with the opened ring to form a double stranded circular DNA in which a DNA fragment  
20 complementary to the DNA or RNA to be detected is inserted (denoted by reference numeral 10 in Fig. 1). The thus obtained double stranded circular DNA is then transfected to a host cell. The double stranded circular DNA is replicated in the host cell, and a first single  
25 stranded circular DNAs 12 containing the DNA fragment complementary to the DNA or RNA to be detected are released from the host cell in the form of a phage.

On the other hand, a double stranded DNA (the DNA may be fragmented with a restriction enzyme, by  
30 ultrasonic treatment or by nick translation or the like) derived from the same phage is labelled with a non-radioactive marker 16 and then denatured to form a second single stranded DNA 18 which is complementary to the region of the first single stranded DNA, which region  
35 is other than the DNA fragment complementary to the DNA or RNA to be detected. The DNA probe of the present

- 9 -

invention may be obtained by hybridizing the first single stranded DNA 12 with the second single stranded DNA 18.

It should be noted that a functional group to which a non-radioactive marker can be attached may be introduced in the double stranded DNA 14 or in the single stranded DNA 18, and after hybridization, the non-radioactive markers may be attached to the functional group.

In the above-mentioned second method of preparing the DNA of the present invention, the second DNA is formed on the region of the first single stranded DNA, which region is other than the DNA fragment complementary to the DNA or RNA to be detected using the region of the first single stranded DNA as a template. This can be accomplished by hybridizing a synthetic DNA (primer) preferably having 10 to 20 bases and more preferably 15 to 17 bases with the 3' end portion of the region of the first single stranded DNA, which region is intended to be made double stranded, and then elongating the primer using Klenow fragment of DNA polymerase in the presence of a nucleotide such as dUTP and dATP to which a non-radioactive marker such as biotin, a hapten, fluorescent substance, chemiluminescent substance can be attached, and in the presence of 4 kinds of nucleotides, i.e., dATP, dCTP, dGTP and dTTP. Whether the second DNA is formed in full or not can be confirmed by electrophoresis using as the control a separately produced standard DNA. If the formation of the second DNA cannot be completed using one primer, two or more primers may be hybridized with the first single stranded DNA.

A preferred embodiment of the second method will now be described referring to Fig. 2. The first single stranded DNA containing a fragment complementary to the DNA or RNA to be detected may be obtained, for example, as in the first method. A synthetic DNA 24 is hybridized with a proper restriction site (EcoRI site in Fig.2) of

- 10 -

the first DNA and at least one synthetic DNAs 22 as a primer is hybridized with the corresponding portions of the first single stranded DNA. Needless to say, the portions of the first single stranded DNA to which the primers are hybridized must be in the region other than the fragment which is complementary to the DNA or RNA to be detected. Thereafter, the DNA is cut with the restriction enzyme. In cases where the DNA probe is used in the circular form, a stopper which terminates the extension of the second chain must be placed at the restriction site instead of the synthetic DNA. Then the primers 22 are extended using DNA polymerase in the presence of dUTP to which allylamine is bonded for the incorporation of an amino group, and in the presence of dATP, dCTP, dGTP and dTTP. To attach biotin to the thus formed second DNA, caproylamidobiotin-N-hydroxysuccinimide ester is reacted with the DNA to obtain a straight chain DNA probe of the present invention.

The DNA probe of the present invention may be used in the form of circle or in the form of straight chain. The DNA probe of the present invention may be used in the same manner as the conventional DNA probes. Thus, a test sample of a tissue, body fluid or the like, which is suspected to contain a virus or a microorganism of interest, or a test sample of a plant or animal cell or a cancer cell is fixed to a glass plate. In the alternative, DNA or RNA extracted from the tissue, body fluid or the cell is fixed on a filter membrane of nitrocellulose or Nylon. Then the glass plate or the filter membrane is then incubated with the DNA or RNA to be detected which has been denatured to single strand. In cases where the DNA probe has a functional group to which a non-radioactive marker can be attached, and does not contain a non-radioactive marker, the non-radioactive marker is attached to the functional group after

- 11 -

hybridization to detect the hybridized probe. Washing is usually conducted in each step.

The present invention may be better understood by referring to the following examples. It should be understood that the examples are presented for the illustration purpose only, and they should not be interpreted in any restrictive manner.

Example 1

1. Preparation of M13mpl9 Single Stranded DNA in Which Adenovirus 2 (Ad2) DNA is incorporated.

In accordance with the method described in "Cloning by Means of M13 and Dideoxy Sequencing Method" published by Amersham Japan on January 1, 1984, a single stranded M13mpl9 DNA in which a Hind III DNA fragment of 5.3 kb of Ad2 DNA was inserted was obtained.

2. Preparation of Biotin-labelled M13mpl9 RF DNA

Five microliters of Solution A4 (0.2 mM each of dATP, dCTP and dGTP) which is a reagent for nick translation, commercially available from BRL Inc., Gaithersburg, Maryland 20877, U.S.A., 2 microliters of M13mpl9 RF DNA solution (0.5 µg/µl, commercially available from Takara Shuzo Co., Ltd., Kyoto, Japan), 2.5 µl of 0.4 mM biotin-11-dUTP, and 35.5 µl of Solution E (H<sub>2</sub>O) were mixed. Then 5 µl of Solution C (0.4 U/µl of BRL DNA polymerase, 40 pg/µl of deoxyribonuclease) was added to the mixture, and the mixture was incubated for 1.5 hours at 15°C. To this reaction mixture, were added 5 µl of Solution D (300 mM EDTA) and 1.25 µl of 5% SDS aqueous solution. This mixture was applied to 5 ml of Sephadex G-50, and was eluted with 1 x SSC (0.15 M NaCl, 15 mM sodium citrate, pH7.0), and eluted fluid was fractioned 150 µl each. Two microliters each of the each fraction was spotted on a nitrocellulose filter and was heated at 80°C for 30 minutes. The filter was immersed in a blocking buffer (PBS (0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>) containing 2% BSA, 0.05% Triton X-100 and 5 mM

- 12 -

EDTA) for 30 minutes at room temperature. The filter was then immersed in a solution of a detection complex "Dete K-1-acp" which is a conjugation of avidin and acid phosphatase, commercially available from Enzo (325 Hudson street, New York, N.Y.), 200-fold diluted with its  
5                   dulation buffer for 1 hour at room temperature. The filter was then washed five times for 5 minutes each with a washing buffer (0.5 M NaCl, 0.5% Triton X-100, 1 mM EDTA, 2% BSA and 10 mM  $KPO_4$ , pH6.5) and then washed twice  
10                   for 2 minutes each with a pre-detection buffer (0.2 M sodium acetate, pH5.8). The filter was incubated at room temperature for 15 hours in a solution which is a 100:1 mixture of a pre-detection buffer solution of 1 mM Naphthol AS-MX phosphate and a pre-detection buffer  
15                   solution of 4 mg/ml of Fastviolet B salt. The colored fractions were combined to obtain a biotin-labelled M13mp19 RF DNA solution of about 1  $\mu$ g/ml.

3. Preparation of DNA Probe Solution (Hybridization Solution)

20                   A solution containing 300 ng/ml of M13mp19 in which Ad2 DNA was inserted, 300 ng/ml of biotin-labelled M13mp19 RF DNA which was denatured by being boiled for 5 minutes, 50% formamide, 4 x SSPE (0.72 M NaCl, 40 mM  $NaPO_4$ , 4 mM EDTA, pH7.4), 5 x Denhardt's solution (0.1%  
25                   polyvinyl pyrrolidone 360, 0.1% Ficoll 400, 0.1% BSA), 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA and 10% dextran sulphate was incubated at 42°C for 16 hours.

4. Detection and Quantification of Ad2 DNA

30                   Five microliters each of the solution of Ad2 DNA (purchased from BRL) with a concentration of 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml and 0.1 ng/ml was spotted on a nitrocellulose filter, and the filter was heated at 80°C for 1 hour. The filter was boiled in physiological saline for 10 minutes and was rapidly cooled, and was  
35                   immersed and incubated in a pre-hybridization solution (50% formamide, 4 x SSPE, 5 x Denhardt's solution, 0.1%

- 13 -

SDS and 0.1 mg/ml denatured salmon sperm DNA) for 3 hours at 42°C. The filter was then incubated in the previously prepared hybridization solution for 19 hours at 42°C, and was washed with 2 x SSC containing 0.1% SDS for 15 minutes at room temperature, twice with the same solution for 15 minutes at 60°C, and once with 2 x SSC which does not contain SDS at room temperature, and was immersed in a pre-detection buffer. The spots on the filter were colored in the same manner as in the preparation of biotin-labelled M13mpl9 RF DNA, and the spots of 10 ng/ml or more of Ad2 DNA were detected.

#### Example 2

1. Preparation of M13mpl9 Single Stranded DNA in Which Ad2 DNA is Inserted

The M13mpl9 Single Stranded DNA in which Ad2 DNA is inserted was prepared by the same manner as in Example 1.

2. Preparation of Biotin-labelled M13mpl9 RF DNA

A mixture of 2 µl of photobiotin solution (1mg/ml) commercially available from BRESA (Adelaide, South Australia, 5001) and 10 µl of PBS was injected in a hematocrit tube. After sealing the both ends of the tube, the tube was placed in ice water and was irradiated with a xenon lamp. The reaction mixture was applied to 5 ml of Sephadex G-50 column, and was eluted with 1 x SSC containing 0.1% SDS. The eluted solution was fractioned 150 µl each. The fractions were subjected to the color test as in Example 1, and the colored fractions were combined to obtain a solution of biotin-labelled M13mpl9 RF DNA of about 1 µg/ml.

3. Preparation of DNA Probe Solution (Hybridization Solution)

The DNA probe solution were prepared in the same manner as in Example 1, except that the biotin-labelled M13mpl9 RF DNA was treated with a ultrasonicator (Kaijo Denki 4280) for 30 seconds at 1A.

4. Detection and Quantification of Ad2 DNA

- 14 -

The detection and quantification were conducted as in Example 1 to detect the spots of the concentration of 10 ng/ml or more.

Example 3

- 5 1. Preparation of M13mpl9 Single Stranded DNA in Which Hepatitis B Virus (HBV) DNA was Inserted (HB/M13)

By the same method as in Example 1, HB/M13 in which a Bam HI fragment of 1.4 kb was inserted was obtained.

2. Preparation of Biotin-Labelled DNA Probe

- 10 1) Formation of DNA on HB/M13

One hundred microliters of an aqueous solution containing 1 $\mu$ g each of 5 kinds of synthetic oligo DNA of 15 bases, one of which being complementary to the Eco RI site of HB/M13, the other being complementary to equally spaced 4 portions of the M13 region of the HB/M13 was  
15 mixed with 40  $\mu$ l of HB/M13 (0.5  $\mu$ g/ $\mu$ l) in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH8.0), and the mixture was incubated at 55°C for 5 minutes. Thereafter, MgCl<sub>2</sub> and NaCl were added to the mixture to final concentrations of  
20 7 mM and 100 mM, respectively. Three microliters of a solution of restriction enzyme Eco RI (12 units/ $\mu$ l) was added and the mixture was incubated at 37°C for 3 hours. Thereafter, 100  $\mu$ l of buffer (67 mM KPO<sub>4</sub> and 6.7 mM MgCl<sub>2</sub>, pH 7.4), 18  $\mu$ l of 1 mM aqueous solution of  
25 Allylamin-dUTP (prepared in accordance with the description in Proc. Natl. Acad. Sci. USA, Vol 78, No. 11, pp.6633-6637, November 1981), and 3  $\mu$ l of DNA polymerase I Large Fragment (4.2 units/ $\mu$ l) were added to the mixture and this mixture was incubated at 25°C for 30  
30 minutes. After phenol extraction, properly double stranded DNA was obtained by ethanol precipitation.

- 2) Labelling with Biotin

The DNA obtained in 1) was dissolved in 100  $\mu$ l of 0.1 M NaHCO<sub>3</sub>, and 20  $\mu$ l of DMSO solution of  
35  $\epsilon$ -caproylamidobiotin-N-hydroxysuccinimide ester (commercially available from BRL) (1mg/ml) was added to



- 15 -

the solution and the mixture was left to react for 10 minutes at room temperature. The reaction mixture was applied to 3 ml of Sephadex G-50 column, and was eluted with 1 x SSC (0.15 M sodium chloride and 0.015 M sodium citrate), and fractions containing DNA were collected.

### 3. Detection and Quantification of HBV DNA

A hybridization solution containing 500 ng/ml of the biotin-labelled DNA probe was prepared as in Example 1. pBR322 vector on which HBV DNA is cloned by a conventional method was cleaved with restriction enzyme Sph I to open the ring, 5  $\mu$ l aliquotes of the solution with concentrations of 1000 ng/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml, respectively were spotted on a nitrocellulose filter. The detection and quantification of HBV DNA which was inserted in pBR322 was conducted with a result that the spot of the concentration of 10 ng/ml or more was positive.

- 16 -

CLAIMS

1. A DNA probe which comprises a single stranded DNA fragment which is complementary to a DNA or RNA to be detected, and a double stranded DNA fragment having a  
5 non-radioactive marker or a functional group to which a non-radioactive marker can be attached.
2. The DNA probe of claim 1, characterized in that substantially the whole region other than the single stranded fragment complementary to the DNA or RNA to be  
10 detected is double stranded.
3. The DNA probe of claim 1 or 2, characterized in that the region other than the single stranded DNA fragment complementary to the DNA or RNA to be detected is originated from a bacteriophage.
- 15 4. The DNA probe of claim 3, characterized in that the bacteriophage is M13.
5. A method of preparing a DNA probe comprising the steps of providing a first single stranded DNA containing a single stranded DNA fragment which is complementary to  
20 the DNA or RNA to be detected; and hybridizing the first single stranded DNA with a second single stranded DNA having a region complementary to a portion of the first single stranded DNA, which portion is other than the DNA fragment complementary to the DNA or RNA to be detected,  
25 the second single stranded DNA having a non-radioactive marker or a functional group to which a non-radioactive marker can be attached.
6. The method of claim 5, characterized in that the region of the first single stranded DNA other than the  
30 DNA fragment which is complementary to the DNA or RNA to be detected is originated from a bacteriophage.
7. The method of claim 5, characterized in that the bacteriophage is M13.
8. The method of any one of claims 5 to 7,  
35 characterized in that the second single stranded DNA has a functional group to which a non-radioactive marker can

- 17 -

be attached, and further comprising the step of attaching the non-radioactive marker to the functional group after the hybridization.

- 5 9. A method of preparing a DNA probe comprising the steps of providing a first single stranded DNA containing a single stranded DNA fragment which is complementary to a DNA or RNA to be detected; and forming a complementary DNA on a region of the first single stranded DNA, which region is other than the single stranded DNA fragment
- 10 complementary to the DNA or RNA to be detected using the region of the first single stranded DNA as a template and using a nucleotide having a non-radioactive marker or a functional group to which a non-radioactive marker can be attached.
- 15 10. The method of claim 9, characterized in that the region of the first single stranded DNA other than the DNA fragment which is complementary to the DNA or RNA to be detected is originated from a bacteriophage.
- 20 11. The method of claim 10, characterized in that the bacteriophage is M13.
12. The method of any one of claims 9 to 11, characterized in that the complementary DNA has a functional group to which a non-radioactive marker can be attached, and further comprising the step of attaching
- 25 the non-radioactive marker to the functional group after the formation of the complementary DNA.

30

35

Fig. 1

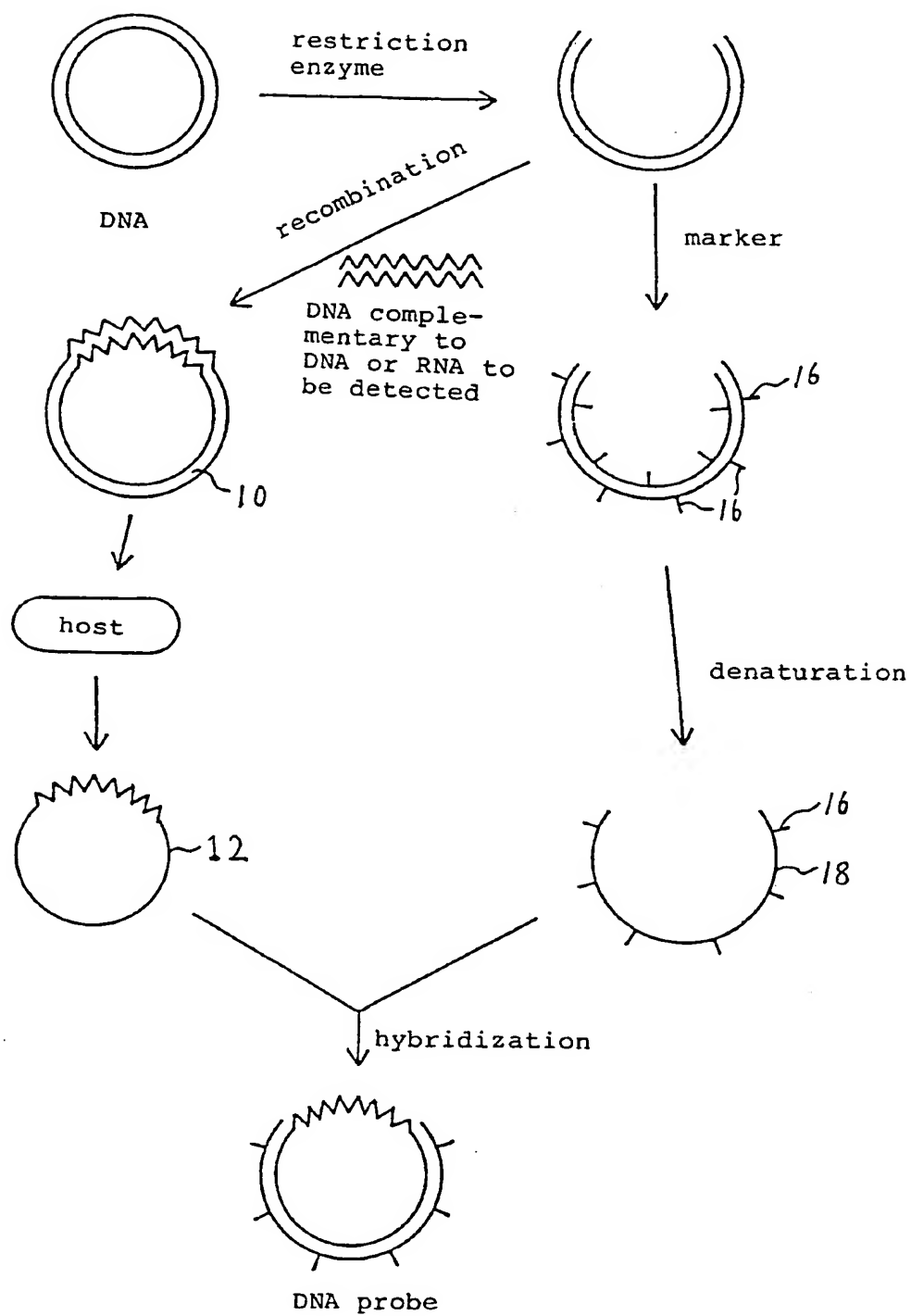
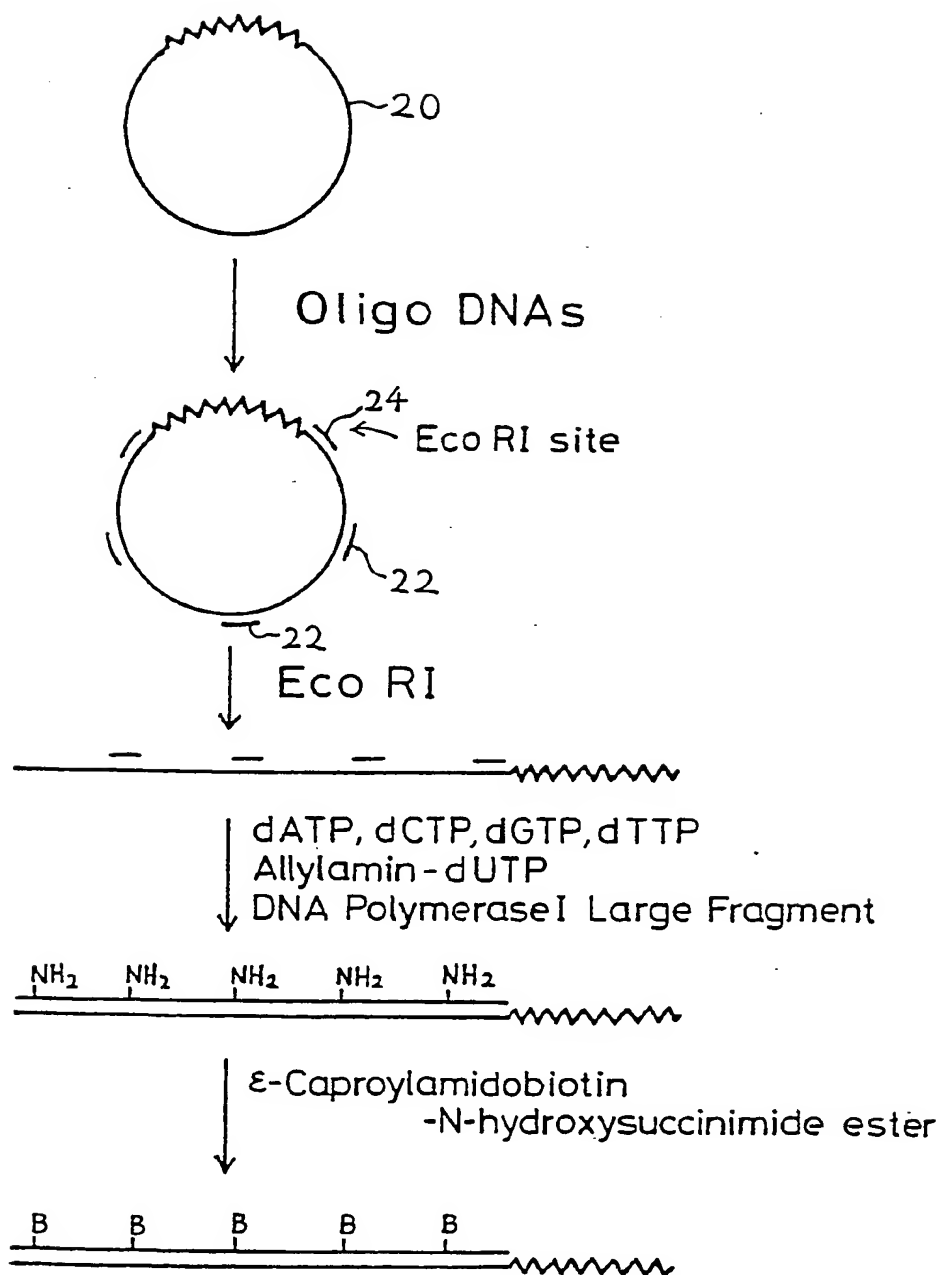


Fig. 2



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/JP 86/00662

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 07 H 21/04; C 12 Q 1/68; C 12 Q 1/70; //  
C 12 N 15/00

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System

Classification Symbols

IPC<sup>4</sup>

C 12 Q; C 07 H

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X,Y	Chemical Abstracts, volume 97, no. 5, 2 August 1982, (Columbus, Ohio, US), N.T. Hu et al.: "The making of strand- specific M13 probes", see page 131, abstract 34130k, & Gene 1982, 17(3), 271-7	1-12
P,Y	EP, A, 0192168 (MOLECULAR DIAGNOSTICS INC.) 27 August 1986 see the whole document, especially page 12, lines 3-14	1-12
Y	EP, A, 0133671 (MILES LABORATORIES INC.) 6 March 1983 see pages 24-26	1-12
Y	EP, A, 0147665 (MOLECULAR DIAGNOSTICS INC.) 10 July 1985 see abstract; page 4, line 5 - page 5, line 31; page 7, line 12 - page 8, line 8; figure 1	1-12
P,A	EP, A, 0172153 (SMITHKLINE BECKMAN CORP.) ./.	

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not  
considered to be of particular relevance

"E" earlier document but published on or after the international  
filing date

"L" document which may throw doubts on priority claim(s) or  
which is cited to establish the publication date of another  
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or  
other means

"P" document published prior to the international filing date but  
later than the priority date claimed

"T" later document published after the international filing date  
or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention

"X" document of particular relevance: the claimed invention  
cannot be considered novel or cannot be considered to  
involve an inventive step

"Y" document of particular relevance: the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art.

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

8th April 1987

Date of Mailing of this International Search Report

19 MAY 1987

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	19 February 1986 see abstract; page 5, line 23 - page 7, line 12	1
	--	
X	EP, A, 0153873 (AMERSHAM INTERNATIONAL plc) 4 September 1985 see abstract, pages 2-5; figure 1/1 -----	1-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON  
-----

INTERNATIONAL APPLICATION NO. PCT/JP 86/00662 (SA 15678)  
-----

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/04/87.

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0192168	27/08/86	AU-A- 5329486	28/08/86
		JP-A- 61195699	29/08/86
EP-A- 0133671	06/03/85	AU-A- 3138784	07/02/85
		JP-A- 60100056	03/06/85
EP-A- 0147665	10/07/85	AU-A- 3652384	20/06/85
		JP-A- 60144662	31/07/85
EP-A- 0172153	19/02/86	AU-A- 4245885	21/11/85
		JP-A- 61001388	07/01/86
EP-A- 0153873	04/09/85	JP-A- 60208997	21/10/85

For more details about this annex :  
see Official Journal of the European Patent Office, No. 12/82